The Study of Epigenetic Changes Associated With Aging In Mammals

A

SYNOPSIS

Submitted for the Partial Fulfillment of the degree of

Doctor of Philosophy

In

ZOOLOGY

Submitted By

Prashansa Ratan

Under the supervision of

Dr. Matteo Pellegrini
Co-Supervisor
Professor

Dr. Amla Chopra
Supervisor
Assistant Professor

DEPARTMENT OF ZOOLOGY, FACULTY OF SCIENCE

DAYALBAGH EDUCATIONAL INSTITUTE (DEEMED UNIVERSITY)

DAYALBAGH, AGRA- 282005

2021

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1. INTRODUCTION

Developmental biologist Conrad H. Waddington in 1942 introduced epigenetics as heritable changes in genetic expression without any alterations in the DNA sequence (Waddington, 2012). Nowadays, it also refers to long-term and stable alterations in the transcriptional potential of a cell that are not necessarily heritable. Epigenetic modifications are stable but can be modulated by physiological and pathological factors and by the environment (Holliday, 2006; Rakyan and Beck, 2006; Whitelaw and Whitelaw, 2006).

Of all the epigenetic mechanisms, DNA methylation, histone post-transcriptional modifications (including methylation, acetylation, ubiquitination and phosphorylation) and RNA interference (RNAi) are thought to be the most functionally important. DNA methylation consists of the addition of a methyl group to the aromatic ring of a single DNA base. It occurs primarily at the cytosine residues of CpG dinucleotides (Razin and Riggs, 1980). It is largely controlled by enzymes known as DNA methyltransferases (DNMTs). Areas rich in CpG sites are typically clustered together known as CpG islands. Hypermethylation of these islands is usually associated with the silencing of gene expression. Inversely, hypomethylation in these regions often leads to gene reactivation (Li and Tollefsbol, 2010; Li et al., 1993). DNA hypermethylation leads to gene silencing and DNA hypomethylation endorses gene activation.

Histone modification involves changes within the basic structure of the chromatin unit, nucleosome. A covalent post-transcriptional modification of histone proteins can impact gene expression by altering chromatin structures. Out of many possible types of histone remodeling (methylation, ubiquitylation, phosphorylation biotinylation, sumoylation, ADP ribosylation and acetylation at the N-terminal group of lysine (K) residues), histone acetylation/deacetylation and methylation are among the most studied and considered to be the most prevalent of the modifications (Kouzarides, 2007; Li et al., 2011; Tollefsbol, 2014).

There are studies that shows interconnection between DNA methylation and histone modification. A large fraction of DNA methylation variability can be explained through the presence of combinations of H3K4me3, H3K4me1, H3K9me3, H3K27me3 and H3K36me3. DNA methylation is generally inhibited by the presence of H3K4me3, H3K4me1 and H3K27me3, while increased levels are found

in regions that are marked by H3K9me3 and H3K36me3(Kai Fu, 2019).

The relationship between epigenetics and aging was proposed many years ago (Table 1) (Calvanese et al., 2009). In young cells, the majority of CpGs within the genome have cytosine methylation. DNA methylation is particularly important during development as it silences the expression of genes in tissues or at the developmental stages when their expression is no longer needed. Since DNA methylation acts as a marker of traits for different organisms. We will focus on the association of DNA methylation with age. There are many facts that shows at least part of the DNA methylation changes during aging are not stochastic but are associated with biological mechanisms involved in the aging process.

Epigenetic age acceleration (i.e. the difference between epigenetic and chronological age) is associated with lung cancer (Levine et al., 2015), cognitive and physical functioning (Marioni et al., 2015), Alzheimer's disease (Levine et al., 2015), centenarian status (Horvath et al., 2015), Down syndrome (Horvath et al., 2015), HIV infection (Horvath et al., 2015), Huntington's disease (Horvath et al., 2016), obesity (Horvath et al., 2014), menopause (Levine et al., 2016), osteoarthritis (Vidal et al., 2016) and Parkinson's disease (Horvath et al., 2016). Many research questions and preclinical studies of anti-aging interventions will benefit from analogous epigenetic clocks in animals (Michael et al., 2016).

Table 1
Epigenetic alteration associated with aging.

Epigenetic hit	Species	Tissue or cell type	References
Global hypomethylation			
	Spawning salmon	Many organs	Berdyshev et al. (1967)
	Rat	Brain, heart	Vanyushin et al. (1973)
	Mouse	Brain, liver, small intestine	Wilson et al. (1987)
	Human	Bronchial epithelial cells	Wilson et al. (1987)
	Human	Leukocytes	Fuke et al. (2004)
	Mouse, hamster and human	Primary cult. fibroblast	Wilson and Jones (1983)
Promoter-specific hypermethylation			
Ribosomal DNA	Rat	Liver and germ cells	Oakes et al. (2003)
ER	Human	Colon	Issa et al. (1994)
MYOD1, N33	Human	Colon	Ahuja et al. (1998)
IGF2	Human	Colon	Issa et al. (1996)
MLH1 and p14ARF	Human	Colon	Issa (2003)
LOX, p16INK4a, RUNX3, TIG1	Human	Gastric mucosa	So et al. (2006)
E-cadherin, c-fos and collagen alpha1	Human	Various tissues	Fraga and Esteller (2007)
Histone marks and histone-modifying enzymes			
Global histone acetylation	Human	Human diploid cells WI-38	Ryan and Cristofalo (1972)
Histone methylation	Rat	Brain and liver	Lee and Duerre (1974)
Senescence-associated heterochromatic foci (SAHF)	Human	Fibroblasts	Narita et al. (2003)
H3K9me, Suv39h1	Mouse	Splenocytes, lymphocytes	Braig et al. (2005)
H3K27me3 and EZH2	Human, mouse	HEF, MEF	Bracken et al. (2007)
H4K20, Suv4–20h	Rat	Liver, kidney	Sarg et al. (2002)
SIRT1	Human, mouse	Human lung, MEF	Sasaki et al. (2006)
SIRT1	Mouse	–	Sommer et al. (2006)

Figure 1: Table showing epigenetic alteration associated with aging (Calvanese et al., 2009).

2. REVIEW OF LITERATURE

Epigenetic factors play a central role in gene regulation and, eventually, in the control of most processes in cells, tissues and the whole organism. Epigenetics has emerged as an important subject area in aging biology and it may be able to explain and/or answer the questions pertaining to the phenotypic changes related to the aging process. Studies showed compelling epigenetic evidence linked to aging from animal, tissue and cell-based models.

Aging is a complex physiological process that results in the compromise of biological functions, increased susceptibility to age-related diseases, and eventually death (Suri et al., 2013). It is well known that both genetic and environmental factors play influential roles in human aging and longevity. With a special emphasis on mammalian aging, there are nine tentative hallmarks that constitute common denominators of aging in different organisms. These hallmarks are-: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al., 2013).

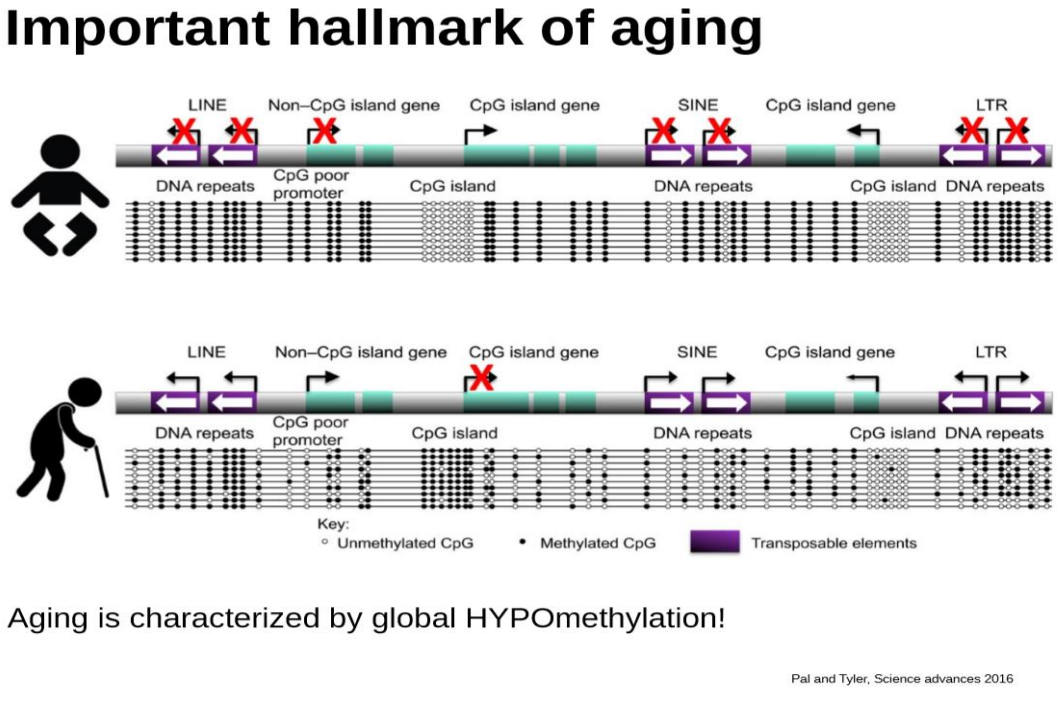

Figure 2: A diagrammatic representation of changes in DNA methylation during aging. Among aging individuals, epigenetic drift leads to unpredictable differences in the methylome (Pal and Tyler, 2016)

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Epigenetics is one of the several components of aging, but its features make it convincing factor for providing the anatomical, physiological, biochemical and genetic evidences for the changes that all

organisms undergo during their lifetime. At least at the cellular level, epigenetics factors are heritable, owing to DNA modification after replication and/or post-translational modification of proteins associated with DNA; for instance, DNA methylation, histone modification or chromatin modeling. Upregulation or downregulation of such modifications at molecular level can in turn contributes to several intracellular events like impaired DNA repair and RNA processing aberrations affecting aging. The modifications in DNA or DNA associated proteins can still be modulated by external factors and therefore represent a molecular link between aging and environment (Calvanese et al., 2009).

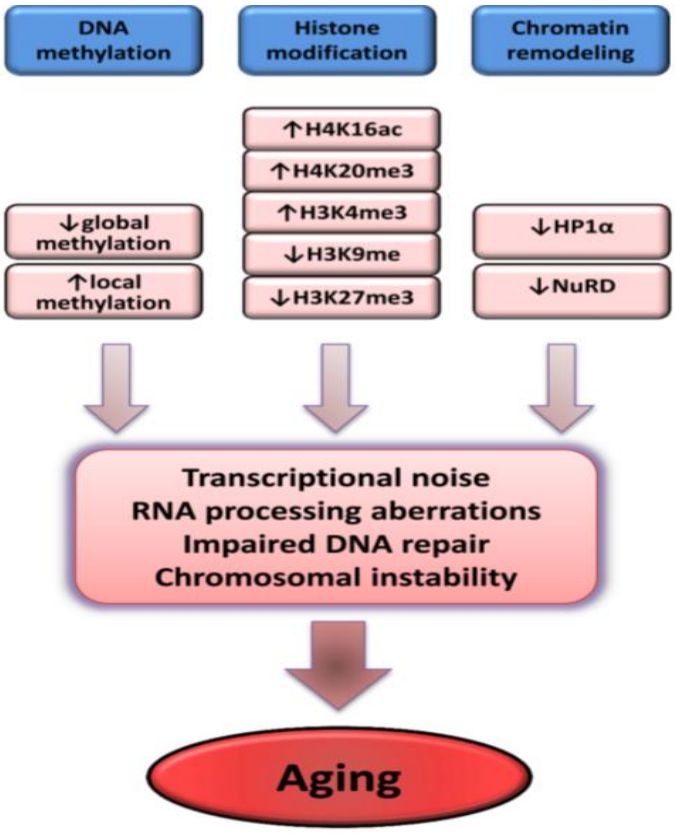


Figure 3: A diagrammatic representation of the alterations in the acetylation and DNA methylation or modifications in histones, as well as other chromatin-associated proteins which can induce epigenetic changes that contribute to the aging process (Lopez-Otin et al., 2013).

It is imperative to study aging process on vertebrate model systems. Till date, however, many commonly utilized aging models have no DNA methylation such as yeast, or limited DNA methylation, such as worms and flies, although they are still useful models to provide some insight into aging process (Sen et al., 2016). Biological limitations to invertebrate models include for instance the anatomy is fundamentally different from mammals. They lack bones and consequently associated

genes that are relevant to understand human aging. For example, Apolipo protein E (APOE) gene and the INK4 locus that in genome-wide studies are associated with high reproducibility to exceptional longevity and age-related diseases (Fortney et al., 2015; Jeck et al., 2012). Thus, these model organisms cannot fully recapitulate the complex nature of aging. It is not possible to consider one single model species for aging, considering the large heterogeneity of animal life spans. Many vertebrate organisms like mouse strains having approximate life span 2-3 years makes it challenging as an efficient laboratory model and providing conclusive comparative study for aging. Though all the studies conducted on vertebrate and invertebrate organisms revealed that although genetic differences and somatic mutations are important factors that underlie longevity, non-genetic contributions also play a major role (Cournil and Kirkwood, 2001).

Canis familiaris are increasingly recognized as a valuable model for aging studies (Boyko, 2011; Kaeberlein et al., 2016). Their lifespan of around 12 years is intermediate between that of mice (2 years) and humans (80 years), which makes it a more realistic model for aging research. They have been used as a model to study multiple human diseases in gene mapping studies. Also, unlike laboratory animals, dogs share the same human environment and lifestyle. They exhibit a wide range of genetic, phenotypic and environmental diversity. These variations are a resource for studying the influential factors that determine the normal aging in dogs. Despite the vast average lifespan difference across breeds, all dogs exhibit a similar developmental, physiological and pathological trajectory (Kaeberlein, Creevy, and Promislow, 2016; Gilmore and Greer, 2015) except that they achieve the progression in aging in many fewer years than humans do. So, the rapidity with which the dogs age, in comparison to humans, provide an advantage in carrying out the investigation of biological mechanisms of aging. This is because the age-related changes can be assessed over a much shorter period (Gilmore and Greer, 2015).

There are many unique aspects of the biology of *Canis familiaris* that makes it relevant for aging studies. Firstly, along with the development of drugs, there is a vast knowledge of dog diseases and many procedures to assess the health and functions of the dogs. Secondly, with the ability to communicate with the humans in a specific way, they represent a unique model for human-like social skills. Thirdly, while humans and dogs diverged early during mammalian evolution, they share recent

evolutionary selection processes and there are studies that shows clear signs of convergent evolution in the human and dog genome (Wang et al., 2013; Axelsson et al., 2013). There are studies proving that the maximum lifespan of dogs is known to correlate with the size of their breed (O'Neill et al., 2013; Speakman 2005; Li et al., 1996). Most Importantly, with the full availability of the sequence assembly of the canine genome, it is well known that that nucleotide sequence of the dog is more similar to the human. More than 650 million base pairs (>25%) of dog sequence align uniquely to the human genome (Kirkness et al., 2003).

The literature reports accurate age estimation methods based on combining the DNA methylation levels of multiple CpG dinucleotide markers (Thompson et al., 2017; Bocklandt et al., 2011; Hannum et al., 2013; Horvath et al., 2013; Lin et al., 2016). Steve Horvath developed a multi-tissue epigenetic age-estimation method (known as epigenetic clock) that combines DNA methylation levels of 353 epigenetic markers known as CpGs (Horvath et al., 2013). An estimate of tissue age (in units of years), which is referred to as "DNA methylation age" or as "epigenetic age" is calculated by the weighted average of these 353 epigenetic markers. DNA methylation age is highly correlated (r=0.96) with chronological age across the entire lifespan (Jung et al., 2015; Gibbs, 2014; Benayoun et al., 2015). The studies have shown that the human epigenetic clock relates to biological age and not to chronological age. We can also safely say that the age acceleration (difference between epigenetic age and chronological age) correlates with longevity.

Whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS) and targeted-bisulfite sequencing are the commonly-used techniques to measure large numbers of random CpGs. The challenge is that such CpGs are often not present in regions conserved across species. Hence to systematically characterize CpGs in regions of the *Canis familiaris* genome, we envisage to perform targeted-bisulfite sequencing for the first time on saliva samples. The targeted-bisulfite sequencing enables high quality evolutionary comparisons of dog methylomes with other mammals. According to a previous study, it has been shown that DNA methylation correlates with age in dogs and wolves as it does in human and related species. This age-dependence of DNA-methylation is conserved at syntenic sites in the respective genomes of these canid species as well as for more distantly related mammalian genomes such as human (Thompson et al., 2017). The bisulfate

sequencing analysis pipeline could be represented diagrammatically as shown below-

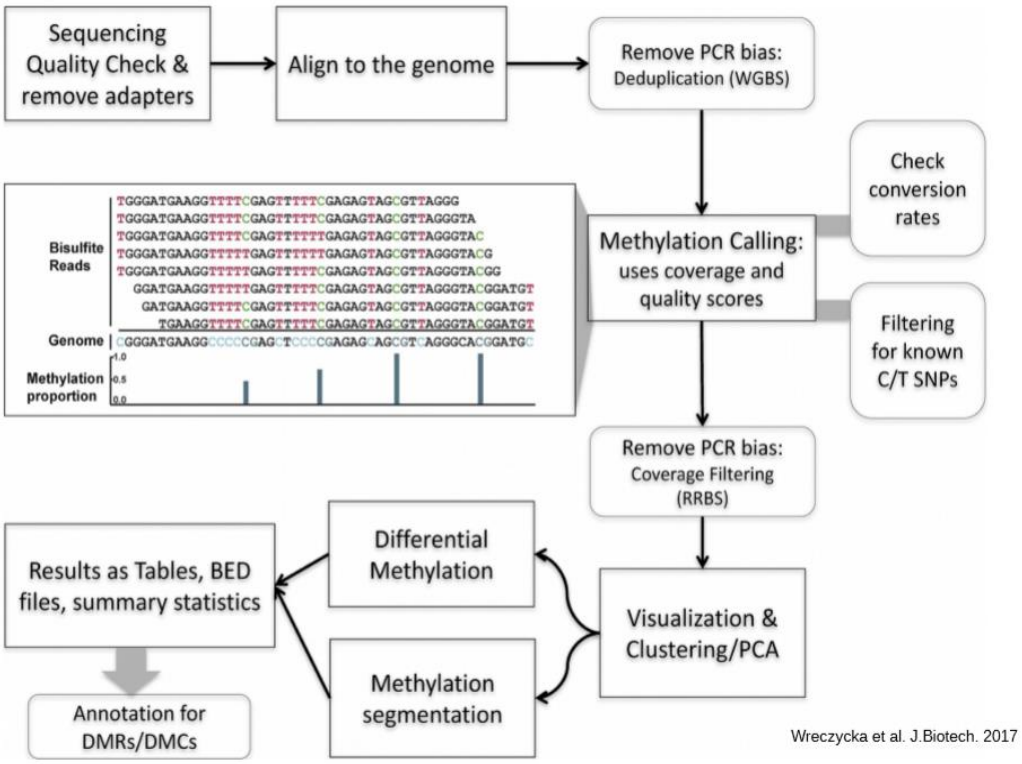


Figure 4: Workflow analysis of DNA methylation using data from bisulfite sequencing experiments (K. Wreczycka et al., 2017).

3. OBJECTIVES

- To establish a DNA methylation based epigenetic clock that allows us to predict the age of *Canis familiaris* from the DNA found in saliva
- To apply the epigenetic clock across breeds to determine whether certain breeds experience epigenetic age acceleration. We hypothesize that small breeds will have lower epigenetic aging rates than larger breeds
- To establish whether we are able to predict the weight of a dog from their epigenome, and to establish whether the deviation of epigenetic and actual weights is associated with breeds.

4. METHODOLOGY

➲ *COLLECTION OF DOG SALIVA SAMPLES*

We will collect buccal swabs from 200 dogs using the Performagene (PG-100) kit from DNA Genotek. A basic questionnaire is designed for the dog owners to better understand the lifestyle of the

dog. Since the study is about the epigenetic aging, the only prerequisite is to know the birth date of the dog (at least the month and year). The questionnaire included the following –

Dog's name-

Dog's birth date-

Dog's breed-

Male/Female-

Spayed/Neutered(Y/N)-

Food Intake of the dog-

Any past known diseases (Rabies/Lyme/Hepatits etc.)-

With the collection of samples, a database is created with the all the information required for the study.

➲ *DNA EXTRACTION FROM SALIVA*

- Incubate the entire sample (in the collection tube) at 50°C for overnight. This incubation step is essential to maximize DNA yield and ensure that nucleases are permanently inactivated. It may be performed at any time after sample is collected and before DNA is purified.
- Spin-down the tubes and transfer the solution into a new tube.
- Add PG-L2P reagent and vortex the sample which releases the DNA from cells. The sample will become turbid as impurities and inhibitors are precipitated.
- Incubate the sample on ice for 10 minutes.
- Centrifuge at room temperature for 10 minutes at 15000 g. Higher centrifugal forces minimize the amount of turbid material that might be carried over into the purified DNA.
- Transfer most of the clear supernatant. Discard the pellet containing the debris and contaminants.
- Add ethanol and NaCl for DNA precipitation. Precipitated DNA may appear as a clot of DNA fibers.
- Sample stands at room temperature for 10 minutes to allow complete precipitation of DNA.
- Centrifuge at room temperature for 10 minutes at 15000 g.
- Remove the supernatant and discard it. DNA pellet (precipitated DNA) should not be disturbed.
- Wash the pellet with 70% ethanol. Remove and discard the supernatant.

- Completely remove the ethanol, being careful not to disturb the pellet and the smear. Carryover of ethanol may impact the performance of the assay.
- Rehydrate the DNA by adding TE solution and by mixing the sample for 1 h at 50°C. Excessive drying of the pellet can make it difficult to rehydrate (dissolve) the DNA and may decrease the yield or make quantification difficult.

➲ *TARGETED BISULFITE SEQUENCING PROTOCOL*

- Library Preparation Fragmentation (using BioruptorSonicator (NEB Next Ultra II Library Prep Kit)

- Quantify the purified DNA with the Qubit BR dsDNA Assay
- Fragmentation using Bioruptorsonicator
- Mix the samples with enzyme mix for End-repair, phosphorylation and A-tailing
- Proceed for pre-methylated adapter ligation
- Continue with SPRI-beads purification
- Samples are pooled together

- Perform Hybridization reaction/ Target capture

- Mix the above fragmented samples with Biotinylated oligonucleotides (received from Integrated DNA Technologies (IDT)) complementary to the regions we need to be enriched
- Incubate the samples overnight for hybridization at 65°C
- Perform washes to capture target DNA-biotinylated oligonucleotides complex with magnetic streptavidin beads
- Perform elution by adding TE buffer and incubating for 5 minutes at 95°C

- Bisulfite Conversion

- Perform Bisulfite Conversion using the Zymo Lightning Methylation kit
- Add Lightning Conversion reagent (RT) to samples
- It is followed by incubation which is followed by purification and desulphonation by adding L-Desulphonation buffer using Zymo spin columns
- DNA is eluted from the Zymo spin columns using TE buffer

- Final PCR amplification
- Mix the samples with the Amplification Primers and DNA Polymerase
- Incubate the samples into the Thermocycler
- PCR amplification is followed by final purification
- Library QC with Qubit BR dsDNA and TapeStation D1000

➲ *BISULFITE SEQUENCE ALIGNMENT*

- Demultiplexing- recovers reads coming from a certain sample based on the specific adapter code
- Convert to fastq files using bcl2fastq or qseq2fstq
- Quality control- checks the quality of the reads before going to the next step
- Trimming- remove adapters, short fragments or trim ends of reads. For trimming, Cutadapt (ability to simultaneously perform task on single-end and paired-end reads) or FASTP will be used (along with simultaneous task performance, preprocesses UMIs and outputs a neat report)
- Remove PCR-product duplicate reads before mapping: Based on sequence similarity and using BS-Seeker2 FilterReads.py script
- Remove PCR-product duplicate reads after mapping: Based on sequence similarity and mapping position using samtools markdup, UMI-tools
- A new platform known as BSBolt will be used for accurately and efficiently mapping the bisulfite-treated reads.

➲ *BISULFITE SEQUENCE ANALYSIS*

- Alignment to the genome- BSBolt tool will be used for the alignment of the reads
- Methylation calling using BSBolt- It will read the methylated data using the BSBolt tool
- Region calls and differential methylation analysis- DNA methylation is bounded (values between 0 and 1) and follow a bimodal distribution. MethyKit, RnBeads or Metilene would be used as tool for fast analysis and defining variable regions creating matrix aggregation (methylation matrix)
- Annotation- To get the annotated regions, in the form of BED file as input, the best and fast way to get output (in the form of regions that are differentially methylated across the genome between different conditions) is GREAT which predicts the functions of cis-regulatory regions
- Visualization- To analyze the DNA methylation from different aspects like visualize regions and

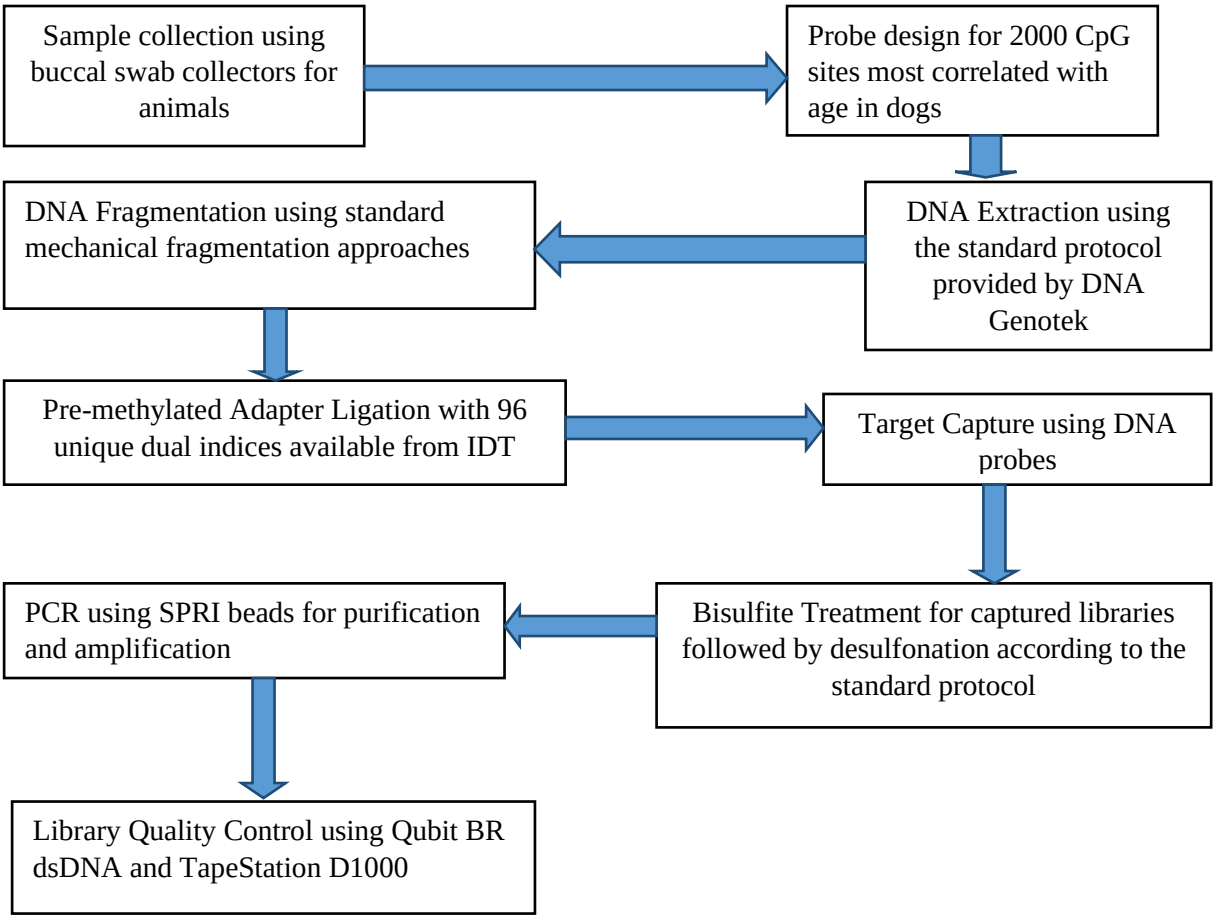
- study the RNA expression and to do EWAS (Epigenome-wide association study)

➲ *DEVELOPING A DOG EPIGENETIC CLOCK*

- The final output of BSBolt will be a single matrix with measures of the methylation levels of all the probe CpG sites across all samples
- In case of the missing data in the matrix, we will use to imputation to estimate the missing values. We will implement K-nearest neighbor (kNN) imputation scheme
- We will test several machine learning approaches including tools like elastic net regression (e.g., glmnet), as well as implementations of support vector machines and random forest classifiers (e.g., scikit-learn) using strict cross-validation to develop an age biomarker that combines CpG sites
- Post model construction, we will evaluate the deviations between the predicted and measured traits.

5. PLAN OF WORK

Part I-


Sample collection using buccal swab collectors for animals
Probe design for 2000 CpG sites most correlated with age in dogs
DNA Extraction using the standard protocol provided by DNA Genotek
DNA Fragmentation using standard mechanical fragmentation approaches
Pre-methylated Adapter Ligation with 96 unique dual indices available from IDT
Target Capture using DNA probes
Bisulfite Treatment for captured libraries followed by desulfonation according to the standard protocol
PCR using SPRI beads for purification and amplification
Library Quality Control using Qubit BR dsDNA and TapeStation D1000

Part II-

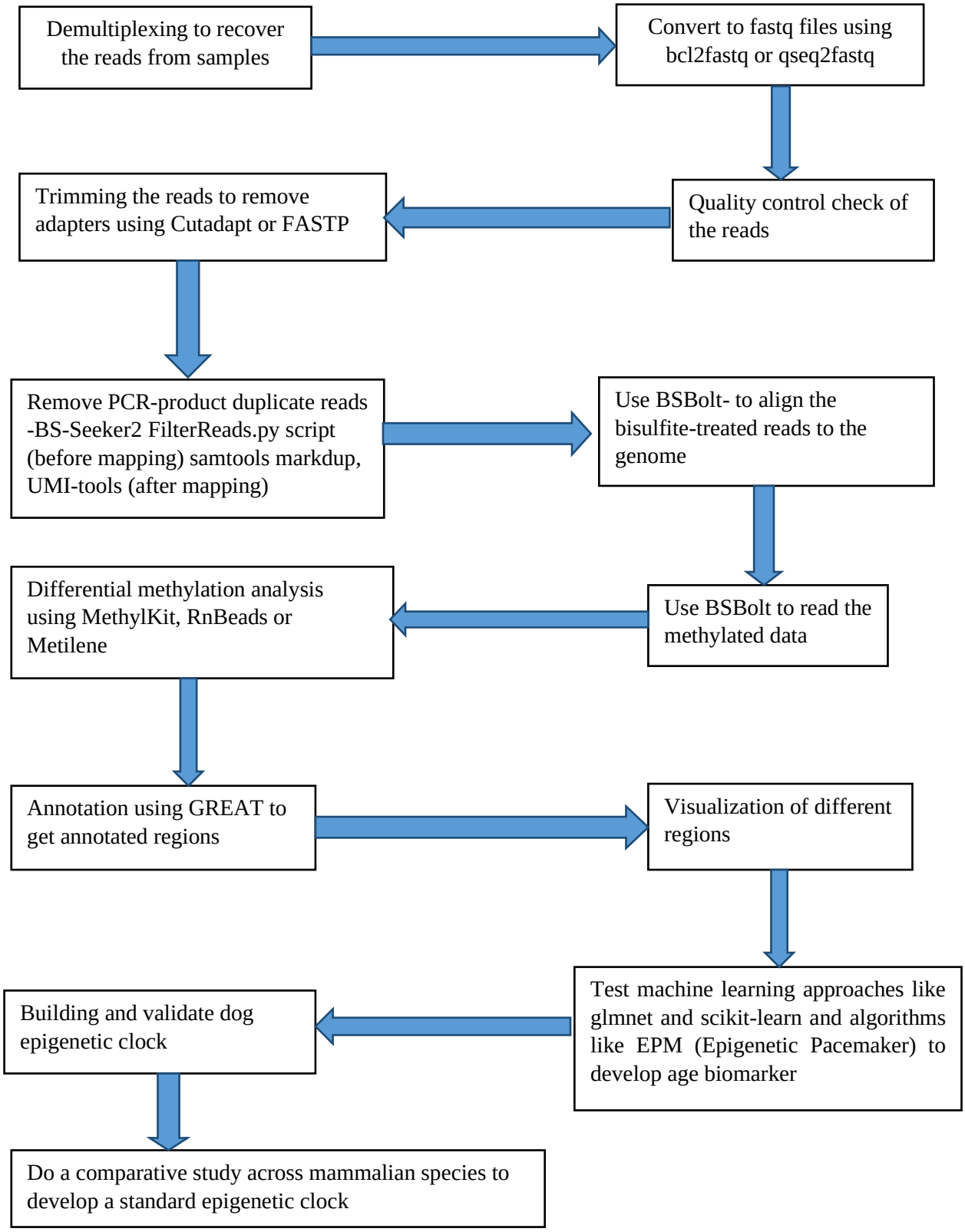

Demultiplexing to recover the reads from samples
Convert to fastq files using bcl2fastq or qseq2fastq
Quality control check of the reads
Trimming the reads to remove adapters using Cutadapt or FASTP
Remove PCR-product duplicate reads -BS-Seeker2 FilterReads.py script (before mapping) samtools markdup, UMI-tools (after mapping)
Use BSBolt- to align the bisulfite-treated reads to the genome
Use BSBolt to read the methylated data
Differential methylation analysis using MethylKit, RnBeads or Metilene
Annotation using GREAT to get annotated regions
Visualization of different regions
Test machine learning approaches like glmnet and scikit-learn and algorithms like EPM (Epigenetic Pacemaker) to develop age biomarker
Building and validate dog epigenetic clock
Do a comparative study across mammalian species to develop a standard epigenetic clock

Figure 5: The proposed plan of work.

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